Characterization of pER371-based *Streptococcus thermophilus–Escherichia coli* shuttle vectors

Native plasmid of *Streptococcus thermophilus* ST137, pER371 (2.7 kb) linearized at various unique restriction sites was individually subcloned into *Escherichia coli* plasmid pUC19 to generate the pUER-series recombinants. A selection cassette consisting of chloramphenicol- and erythromycin-resistance genes was spliced into each construct to generate the pMEU shuttle vectors. Electrotransformation of *Streptococcus thermophilus* with these vectors showed that a ca. 1.7 kb *Bst*EII/*Ban*II fragment is essential for plasmid replication. A shuttle vector, pMEU14′-1 (5.3 kb), was constructed using the minimally required fragment for replication. A chloramphenicol acetyltransferase (*cat*) gene was successfully expressed in the ultimate *S. thermophilus* host by using pMEU14′-1. Cloning vectors derived from pER371 should provide valuable alternative gene delivery vehicles for the genetic engineering of lactic acid bacteria.

Introduction

Streptococcus thermophilus is an important starter culture used in the fermentation of yogurt and certain cheeses. Genetic engineering approach provides a potentially powerful means for strain improvement of this microbe for the purpose of developing novel dairy products. Consequently, plasmid vectors necessary for the delivery of exogenous genes into this microorganism have been the subject of many studies. Most studies centered on the use of heterologous vectors for carrying foreign genes into *S. thermophilus* (Mercenier, 1990; Constable and Mollet, 1994; Jacobs *et al.*, 1995; Moineau *et al.*, 1995; Mollet *et al.*, 1993; Somkuti *et al.*, 1991, 1993; Vaughan *et al.*, 1996). In the interest of developing food-grade vectors, cloning vehicles based on a cryptic plasmid, pER8, of *S. thermophilus* were constructed (Somkuti and Steinberg, 1986a). These vectors had been used to express various heterologous genes in *S. thermophilus* by using promoters obtained either synthetically (Somkuti *et al.*, 1995) or via molecular cloning (Solaiman and Somkuti, 1995).

S. thermophilus harbors a spectrum of plasmids with different sizes and DNA-homology attributes (Somkuti and Steinberg, 1986a, Somkuti and Steinberg, 1991). In this communication, we report the use of a small cryptic plasmid, pER371 (2.7 kb), to construct new cloning vectors. Since pER371 and pER8 belong to different plasmid DNA homology groups, vectors derived from them are potentially useful for complementary cloning applications.

Materials and methods

Bacterial strains, plasmids, and growth conditions

E. coli DH5α (BRL Life Technologies, Gaithersburg, MD) and *S. thermophilus* ST128 and ST137 (laboratory collection) were grown in Luria-Bertani (LB) and tryptone-yeast extract-lactose broth, respectively, as previously described (Somkuti and Steinberg, 1986a). Ampicillin (100 μg/ml), chloramphenicol (15–35 μg/ml) and erythromycin (200 μg/ml) were added as needed to *E. coli* medium. *S. thermophilus* transformants were selected in growth medium containing erythromycin (10–15 μg/ml) and/or chloramphenicol (5 μg/ml).

Vectors pUC18/19 were from BRL Life Technologies. pER371 was the smaller of the two resident plasmids in *S. thermophilus* ST137 (Somkuti and Steinberg, 1986a).

Molecular cloning procedures

Restriction endonucleases, DNA-modifying enzymes and T4 DNA ligase were purchased from and used according to the specifications of the following suppliers: BRL Life Technologies, New England BioLabs (Beverly, MA), and United States Biochemical (Cleveland, OH). Competent *E. coli* DH5α was transformed with plasmid DNA by a heat-shock protocol detailed by the vendor. Method for the electrotransformation of *S. thermophilus* had been described elsewhere (Somkuti and Steinberg,

1988). Plasmids were purified from *E. coli* (Solaiman and Somkuti, 1993) and *S. thermophilus* (Somkuti and Steinberg, 1986b) according to previously published methods. Agarose gel electrophoresis of DNA was performed in TBE buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA). DNA fragments were electroeluted as needed from agarose gel using a SixPac GE200 Eluter (Hoefer Scientific Instrument, San Francisco, CA). When warranted, further purification and/or concentration of nucleic acid samples was performed using Elutip-d columns (Schleicher & Schuell, Keene, NH).

Assay of cat activity in transformed *S. thermophilus*

Overnight cultures (5 ml) of *S. thermophilus* transformants containing pMEU14′-1 and pMEU14′1*cat* were harvested by centrifugation and resuspended in 0.5 ml of POM buffer (50 mM K-PO_4; 1 mM $MgCl_2$; pH 7.4). Cells were permeabilized by adding 25 µl of acetone/toluene mixture (9:1; v/v), followed by incubation at room temperature for 20 min with intermittent vortex mixing. Permeabilized cell suspensions (PCS) were kept on ice until use, and stored in –70°C freezer for long term storage.

Chloramphenicol acetyltransferase activity of the PCS was assayed by using a *FAST* CAT kit supplied with green fluorescent BODIPY FL 1-deoxychloramphenicol (deoxy-Cm) substrate (Molecular Probes, Inc., Eugene, OR). Assay was performed by preincubating a mixture of 300 µl PCS and 10 µl BODIPY FL deoxy-Cm substrate at 37°C for 5 min. Freshly prepared 9 mM acetyl-CoA (50 µl) was added, and the reaction mixture was incubated for 17.5 h at 37°C with 250 rpm rotary shaking. Product and unreacted substrate were extracted with 2 aliquots (600 µl and 500 µl) of ice-cold ethyl-acetate. The combined organic phase was evaporated to dryness in a RotoVac, and the final residues were dissolved in 15 µl ethyl acetate. The entire suspension was spotted in 3 5-µl aliquots on a thin-layer-chromatography plate (Pre-coated SIL G-25 TLC plate, 0.25 mm without gypsum, Brinkman Instruments, Inc., Westbury, NY). Methanol/chloroform (15:85; v/v) was used to develop the plate.

The chromatogram was visualized under UV light transillumination.

Results and discussion

Construction of pUER and pMEU plasmids

The construction of pUER-series plasmids is depicted in Fig. 1. The *S. thermophilus* cryptic plasmid, pER371,

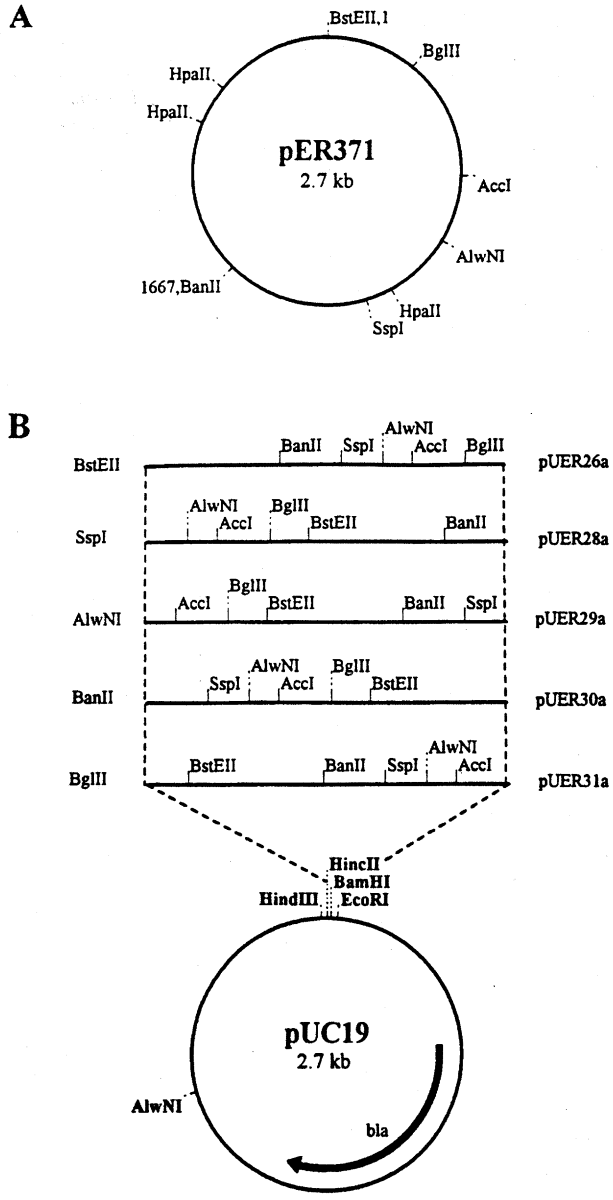

Figure 1 The construction of pUER plasmids. Panel A: pER371 restriction map. Panel B: Configuration of various pUER constructs. Restriction enzymes used to linearize pER371 were shown on the left hand side. For simplicity, not all restriction sites for any particular endonuclease were shown.

was linearized at unique restriction site *Bst*EII, *Ssp*I, *Alw*NI, *Ban*II or *Bgl*II, and separately subcloned into the *Hinc*II site of pUC19 by blunt-end ligation to produce the corresponding pUER plasmids. For each combination, two constructs representing both possible orientations of the insert were obtained from the *E. coli* transformants, indicating that the subcloned pER371 sequence did not interfere with the pUC19 replication

Table 1 Origin and *S. thermophilus* electrotransformability of pMEU vectors

Vector	*Parental Plasmid*	*S. Thermophilus Transformation*
pMEU14a	pUER26b	+
pMEU15b	pUER28a	–
pMEU16a/b	pUER29a	–
pMEU17b	pUER30a	+
pMEU18a	pUER29b	–
pMEU19a	pUER31a	–

function. The pUER plasmids appeared to be stable in *E. coli*, as no apparent deletions were observed.

To test whether the various subcloned pER371 sequences were capable of supporting plasmid replication in *S. thermophilus*, the pUER recombinants were equipped with antibiotic-resistance markers to allow for selection of transformants. The *cat,erm* cartridge (2.5 kb) previously used for the construction of pER8-based shuttle vectors (Solaiman and Somkuti, 1993) was employed here. The cartridge was excised from pUCE1 (Solaiman and Somkuti, 1993) by *Eco*RI digestion and directly spliced into the unique *Eco*RI site of pUER plasmids. The chimeric plasmids, designated pMEUs, were isolated from *E. coli* transformants selected first for ampicillin-resistant (100 μg/ml), and then screened for resistance to chloramphenicol (35 μg/ml) and erythromycin (200 μg/ml). Attempts to directly select transformants on solid growth medium containing chloramphenicol and erythromycin failed to yield any clones, in agreement with previous observations that the two resistance markers were poorly expressed in *E. coli* (Solaiman and Somkuti, 1993). The isolated pMEU vectors with the *cat* determinant aligned with the *bla* gene were assigned a suffix **a**; constructs with the *cat* and *bla* genes opposing each other were given a suffix **b**. In some instances, only **a** or **b** configuration was isolated, suggesting that the orientation of *cat,erm* cartridge in the final construct affects its segregational stability. Table 1 summarizes the origin of pMEU plasmids used to define replication-essential region of pER371.

Defining replicon of pER371

When *S. thermophilus* ST128 was electrotransformed with the pMEUs shown in Table 1, only the 14a and 17b constructs (Fig. 2) yielded Em^R transformants. The transformation frequencies were estimated as 70 and 250 c.f.u./μg DNA for pMEU14a and pMEU17b, respectively. These results indicate that the replication function of pER371 is located within a ca. 1.7 kb region

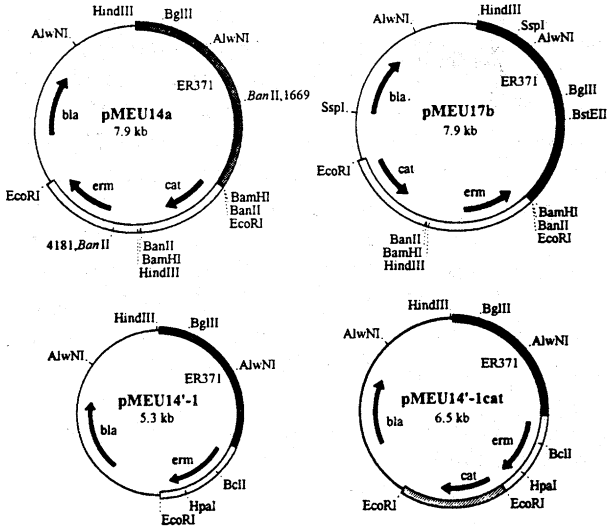

Figure 2 Restriction maps of plasmids. For restriction enzymes with multiple cut sites, not all of the restriction sites are shown. The dotted-box region represents sequence originated from pER371, the open-box region denotes sequence derived from *cat,erm* cartridge of pUCE1 (Solaiman and Somkuti, 1993), and the hatched-box region indicates the *cat* cassette from pUCM910 (Solaiman and Somkuti, 1993). The italicized *Ban*II sites (at nucleotide numbers 1669 and 4181) on the pMEU14a map mark the delineating points of the 2.5-kb fragment removed during the construction of pMEU14′-1.

flanked by the *Bst*EII and *Ban*II sites at coordinates 1 bp and 1667 bp, respectively (Fig. 1). Consequently, linearization of pER371 at *Bgl*II, *Alw*NI and *Ssp*I sites disrupted the replication function, resulting in pMEU constructs incapable of replicating in *S. thermophilus* (Table 1). Plasmid preparations obtained from Em^R *S. thermophilus* contained only intact pMEU14a and pMEU17b, indicating that these constructs did not undergo deletion in the host.

Construction and utilization of pMEU14′-1 shuttle vector

Since electrotransformation frequency for *S. thermophilus* is inversely related to plasmid size (Somkuti and Steinberg, 1988), the pMEU14a construct was modified to yield a smaller size vector. In this undertaking, pMEU14a (Fig. 2) was digested with *Ban*II, and the largest fragment containing the essential replication region of pER371 was purified by agarose gel electrophoresis and electroelution. Recircularization of this fragment yielded pMEU14′-1 plasmid (5.3 kb, Fig. 2) which electrotransformed *S. thermophilus* with an improved transformation frequency of ≥300 c.f.u./μg DNA.

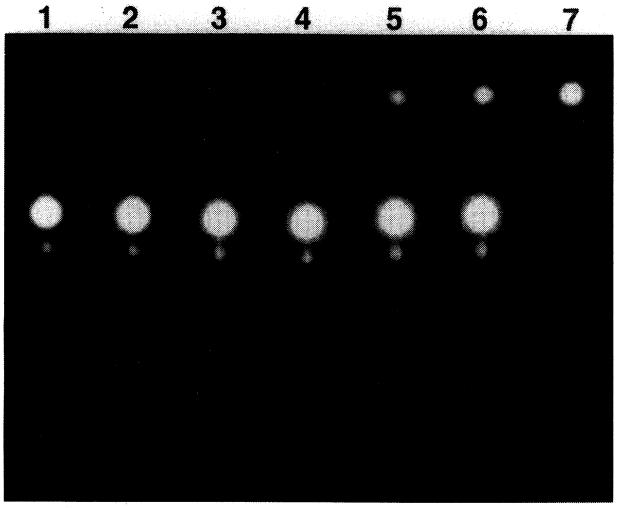

Figure 3 Chloramphenicol acetyltransferase activity in *S. thermophilus* transformants. Three hundred microliters of 0.1 M Tris.HCl, pH 7.4 (lane 1), permeabilized *S. thermophilus* ST128 host (lane 2), ST128 transformed with pMEU14′-1 vector (lanes 3 and 4), and ST128 transformed with pMEU14′-1*cat* plasmid (lanes 5 and 6) were used in the reaction mixtures. The green fluorescent BODIPY FL 3-acetyl-1-deoxychloramphenicol reference standard (Molecular Probes, Inc.) was chromatographed in lane 7.

The usefulness of pMEU14′-1 as a shuttle vector was demonstrated by its ability to carry and express a heterologous *cat* gene in *S. thermophilus*. For this purpose, a *cat* gene cassette was excised from pUCM910 plasmid (Solaiman and Somkuti, 1993) by *Eco*RI digestion and spliced into the *Eco*RI site of pMEU14′-1 to yield pMEU14′-1*cat* plasmid (Fig. 2). Introduction of pMEU14′-1*cat* into *S. thermophilus* ST128 by electrotransformation allowed for the isolation of chloramphenicol-resistant clones at an average transformation frequency of 55 c.f.u./μg DNA. Plasmid screening of these transformants showed the presence of the 5.3-kb pMEU14′-1*cat* (data not shown). Permeabilized cells prepared from these transformants were capable of catalyzing the conversion of fluorescently labeled Cm to its acetylated derivative (Fig. 3), indicating that the heterologous *cat* gene was expressed. These results showed that pMEU14′-1 shuttle vector could serve as an alternative heterologous gene expression vehicle for use in the genetic engineering of *S. thermophilus* and possibly other lactic acid bacteria.

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